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FULL LENGTH ARTICLE

A simple protocol for preparation of a liposomal vesicle with encapsulated plasmid DNA that mediate high accumulation and reporter gene activity in tumor tissue

Torben Gjetting [a,b,*], Thomas Lars Andresen [b], Camilla Laulund Christensen [a], Frederik Cramer [a], Thomas Tuxen Poulsen [a], Hans Skovgaard Poulsen [a]

[a] Department of Radiation Biology, section 6321, Finsen Center, Copenhagen University Hospital, Copenhagen, Denmark
[b] Department of Micro- and Nanotechnology, Technical University of Denmark, Kgs. Lyngby, Denmark

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ABSTRACT

The systemic delivery of gene therapeutics by non-viral methods has proven difficult. Transfection systems that are performing well *in vitro* have been reported to have disadvantageous properties such as rapid clearance and short circulation time often resulting in poor transfection efficiency when applied *in vivo*. Large unilaminary vesicles (LUV) with encapsulated nucleic acids designated stabilized-plasmid-lipo-particle (SPLP) have showed promising results in terms of systemic stability and accumulation in tumor tissue due to the enhanced permeability and retention effect (EPR). We have developed a simple protocol for the research-scale preparation of SPLPs from commercially available reagents with high amounts of encapsulated plasmid DNA. The SPLPs show properties of promising accumulation in tumor tissue in comparison to other organs when intravenously injected into xenograft tumor-bearing nude mice. Although transcriptionally targeted suicide gene therapy was not achieved, the SPLPs were capable of mediating reporter gene transfection in subcutaneous flank tumors originating from human small cell lung cancer.

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1. Introduction

In the field of gene therapy, efficient gene delivery *in vivo* based on non-viral methods remains a major challenge, with an overwhelming variety of polymeric and liposomal compounds being tested [1]. A major obstacle has been the fact that extremely efficient methods involving cationic liposomes for gene delivery to cells *in vitro*, perform very poorly when tested in animals [2]. Although a regime of transfection-potent lipoplexes has been established *in vitro* [3], *in vivo* applications require different physical–chemical properties and only limited information about these have been described.

The development of liposomal carriers with enhanced systemic stability has mainly been advanced by the liposomal formulation of chemotherapeutics, i.e. doxorubicin into DOXIL® that is FDA-approved for use against several cancers [4]. Here a great advantage of therapeutic efficiency over the naked drugs has been accomplished [5]. A great accumulation in disease area, i.e. tumor tissue due to the so-called enhanced permeability and retention effect (EPR) is a hallmark of these liposomal formulations [6] where the property of long circulation is accomplished by a 5–10% PEG polymers screen on the liposomal surface.

Furthermore, efficient encapsulation of plasmid DNA in liposomes can be achieved using an ethanol-mediated condensation procedure [7,8], and this was established in our laboratory [9]. The technology of stabilized plasmid lipo-particles (SPLPs) has progressed in recent years [10] and we decided to investigate these methods for laboratory scale studies of a gene therapy strategy in mice using conventional lipid reagents, hence we included a tritium-labeled lipid in the formulation enabling evaluation of systemic circulation and biodistribution of SPLPs [11]. A robust laboratory-scale protocol allows for researchers to perform experiments investigating the biological properties of SPLPs and the interaction with the biological milieu in order to characterize the barriers to successful gene delivery.

Aiming at gene therapy of small cell lung carcinoma (SCLC) [12] we have recently showed high and specific effect of a suicide gene therapy system [13]. At the time of diagnosis SCLC often appears disseminated to various extra-thoracic organs [14], and therefore a systemic distribution of the therapeutic agent is

Abbreviations: SPLP, Stabilized plasmid–lipid particle; PEG, Polyethylene glycol; SCLC, Small cell lung carcinoma; EPR, Enhanced permeability and retention effect; PDI, Polydispersity index; SCD, Super cytosine deaminase

* Corresponding author. Present address: Department of Micro- and Nanotechnology, Technical University of Denmark, Ørsteds Plads, Building 345B, 2800 Kgs. Lyngby, Denmark. Tel.: +45 4525 8169; fax: +45 4588 7762.
E-mail address: torben.gjetting@nanotech.dtu.dk (T. Gjetting).

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demanded. Hence in the current study we have exploited the potential of transcriptionally targeted suicide gene therapy using SPLPs as a delivery vehicle for systemic treatment of a mouse model of SCLC.

2. Materials and methods

2.1. Materials

All chemicals, e.g. synthetic cholesterol were purchased from Sigma-Aldrich Inc. (Brøndby, Denmark) unless otherwise stated. DDAB: Dimethyl-dioctadecyl-ammonium bromide, DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and DSPE-PEG2000 (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) [1,2-3H]-Cholesteryl Hexadecyl Ether (^{3}H-CHE) was purchased from Perkin Elmer (Skovlunde, Denmark). High quality plasmids; pCMV-LUC (sequence available upon request) and pEGFP-N1 (Clontech, Mountain View, CA, USA) preparations were made with the Endo-free Giga kit from Qiagen GmbH (Hilden, Germany) according to the manufacturer's instructions. Glassware used for lipid work was washed and rinsed in mQ water, baked at 180 °C for 6 h and de-dusted by pressurized air prior to use. Lipid solutions in chloroform were handled with gastight glass syringes (Hamilton, VWR, Herlev, Denmark) reserved for this purpose. Syringes were rinsed with chloroform and 20% EtOH in water only.

The H1299 and NCI-H69 cell lines (obtained from ATCC, Boras, Sweden) were cultivated in RPMI medium supplemented with pen-strep and 10% fetal calf serum (Invitrogen Inc., Taastrup, Denmark). Six-week old male NMRI mice were from Taconic Europe (Lille Skensved, Denmark) and housed at Department of Experimental Medicine, University of Copenhagen. All animal experiments were performed according to ethical guidelines and under valid license from the Danish Animal Experimentation Board.

2.2. Methods

2.2.1. Preparation of SPLPs: Cholesterol/DSPC/DDAB/DSPE-PEG2000 liposomes encapsulating plasmid DNA

Chloroform solutions of lipids (10–20 mg/ml) were mixed in a 12×75 mm^2 glass tube (Thermo Fischer Scientific, Slangerup, Denmark) at the following composition (20 μmol total lipid, mole percent): Cholesterol 55%, DSPC 20%, DDAB 15% and DSPE-PEG2000 10%. In experiments where a radioactive label was used ^{3}H-CHE (50 μCi, 50 Ci/mmol in toluene) was added. The solvent was evaporated under vortexing and under a thin nitrogen gas stream allowing a thin, fairly even lipid film to form on approximately 6 cm of the glass surface. High vacuum was applied overnight to ensure complete solvent evaporation. A Tris–HCl buffer (300 μl, 50 mM, pH 7.0) was used to hydrate the lipids and allow for vesicle formation. The tube was rotated and lipids allowed to hydrate overnight at room temperature. The next day the liposome preparation was placed in a metal basket and sonicated for 2 min using a Bransonic water bath (MT-1510, 42 kHz, 80 W, setting "sonics", Branson Ultrasonics, Danbury, CT, USA). Plasmid DNA (Endo-free GIGA prep, 200 μg, 5.7 μg/μl in Tris-buffer) was added to the tube and after collecting the material at the bottom of the tube by a brief spin exactly one volume of 80% ethanol in Tris-buffer (50 mM, pH 7.0) was added dropwise and with mixing during one minute.

The tube was closed and subjected to five cycles of freeze-thaw between dry ice/EtOH and 37 °C water bath with 2–3 min in each step. Liposomes were downsized using 11 passes in a hand-held, small-scale extruder (Avestin Europe GmbH, Mannheim, Germany) with polycarbonate nucleopore filters (400 nm, 200 nm and 100 nm, Whatman, Frisenette, Knebel, Denmark). For each step a small volume of buffer to wash the extruder ensure a complete liposome recovery. The entire SPLP volume (typically 1 ml) after the extrusion process was transferred to a dialysis cassette (PIERCE, Thermo, 10 kDa MWCO) and dialyzed against 0.5 l HEPES buffer (pH 7.4) overnight at room temperature with one buffer exchange.

MALDI-TOF mass spectrometry analysis of liposome preparation made at 20 μmol scale confirmed the lipids in the composition and did not reveal any degradation (data not shown).

2.2.2. Characterization of SPLP

Plasmid encapsulation and ability to migrate in an electrical field was investigated using agarose gel electrophoresis [9,15,16]. Samples of SPLP from different stages of the encapsulation procedure were loaded on a standard 1% agarose–Tris–Borate–EDTA gel containing 2 μg/ml ethidium bromide. After completion the gel was photographed under UV light. Subsequently, the concentration of plasmid DNA in liposome was determined using a variation of the PicoGreen assay (Invitrogen) as described by Jeffs et al. [7]. A typical dose for intravenous injection contained 20 μg DNA and 4 μmol lipid in 200 μl HEPES buffer.

A Zetasizer Nano ZS (Malvern Instruments Inc., Malvern, UK) was used for characterizing the particle size by dynamic light scattering. Preparations of liposomes were diluted to approximately 1 mM total lipid and placed in a clear disposable zeta cell (Malvern). Size was determined using 4 cycles of 3 min. at standard settings for vesicles and with "general purpose" parameter settings. The quality of size measurements given as the volume-weighted mean diameter were analyzed by evaluating polydispersity index (PDI), scattering correlation and cumulants fit. Subsequently, samples were analyzed for zeta potential of particles using standard settings with three repeated measurements of 20 zeta runs and assessing the quality of measurements by evaluation of the phase plot.

2.2.3. Gene expression analysis in vitro

Adherent H1299 were plated the day prior to the experiment in 6-well plates, 300,000 cells per well. NCI-H69 cells growing in suspension were single-cell resuspended on the day of the experiment and counted in a hemocytometer using Trypan Blue (0.4%) staining to discriminate from dead cells before placing 2×10^6 cells in 6 well plates. Forty microlitres (2–4 μg/0.8 μmol) of plasmid DNA/liposome preparation was added to cells in full growth medium and incubated for 2 days at 37 °C before analysis of reporter activity. Here, cultured cells were washed with phosphate-buffered saline (PBS) and lysed in 100 μl passive lysis buffer (Promega Inc., Madison, WI, USA) for 10 min. After centrifugation for 1 min, the supernatant was analyzed for luciferase activity (20 μl, Luciferase kit, Promega) using a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany) and total protein concentration (20 μl, 10 times diluted, BCA kit, Pierce/Thermo, Rockford, IL, USA) using an OpsysMR microplate reader (Dynex Technologies GmbH, Berlin, Germany). Using a purified, recombinant firefly luciferase (Promega) for standardization, luciferase activity was expressed as picogram luciferase enzyme per milligram of total protein (pg luc/mg protein).

2.2.4. SCLC tumor model establishment and evaluation of growth

The SCLC xenograft model was established as previously described [13,17]. Briefly, 5×10^6 NCI-H69 cells per flank were injected subcutaneously into 6–8-week old male nude NMRI mice. Tumors from injected mice (termed passage 0) were used for serial transplantation of mice that entered experimental

protocols (passage 1) or used for serial transplantation of new animals (passage 2). Xenograft tumors used in the experiments were passaged up to 4 times. The tumor growth was monitored by measuring with a caliper the two perpendicular diameters and the tumor mass was calculated as previously described [13]. For suicide gene therapy experiments following intravenous injection of SPLPs, an intraperitoneal dose of 5-fluoro-cytosine (500 mg/kg) was given the same and the next day when animals were euthanized after two days for biodistribution measurements [13].

2.2.5. Pharmacokinetics, luciferase activity and biodistribution in vivo

Tumor-bearing male NMRI nude mice were injected in the lateral tail vein with 200 μl SPLP (20 μg DNA and 4 μmol lipid) prepared as described above. One or two days later animals were euthanized by cervical dislocation and organ samples (tumor, heart, lung, liver, kidney, spleen and tail (1 cm upward of injection site); 20–150 mg) were isolated and snap frozen. Organ samples were mixed with 1 ml passive lysis buffer (Promega Inc.) supplemented with Protease Inhibitor Cocktail Set III (Merck Chemicals, Glostrup, Denmark) ground in a ball mill (Qiagen) using one steel ball (5 mm) and shaking for 6 min. After centrifugation for 10 min at 4 °C the supernatant was isolated and luciferase activity and protein concentration was measured as described previously.

Using the lipid marker ^{3}H-CHE [11,18], tritium-labeled DNA/lipoplexes with varying degree of PEGylation were injected in a single dose (100 μl) containing approximately 1 μCi tritium label. In these experiments half of the homogenate (500 μl) was isolated for scintillation counting before centrifugation. If more than 90% of the counts were found in the tail sample the injection was considered as failed and the mouse was excluded from the experiment. Relative distribution of counts in different samples was calculated as CPM per g tissue sample. For calculation of the total radioactivity in each organ, a relative organ weight in tumor-bearing nude NMRI mice per gram body weight was determined from ten mice with a standard deviation less than 4%. Using this tabulation the total organ weights in mice could be estimated in the experiments. Hence the radioactivity accumulating per gram organ was expressed relative to the injected dose.

Blood samples (100 μl) were drawn from the eye by periobital plexus puncture after 15 min, 2 h, 5 h and 24 h and immediately mixed with 10 ml scintillation liquid (UltimaGold, Perkin Elmer, Skovlunde, Denmark) and counted in a Beckman LS 6500 liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA). After the last blood sampling the animals were euthanized and organs sampled as described above.

2.2.6. Detection of plasmid in mouse tissue samples

Plasmid and chromosomal DNA in homogenates from 5 to 10 mg tissue were purified using a Gentra Puregene Blood Kit (Qiagen) and subjected to PCR analysis with the following conditions: Platinum Taq DNA polymerase (1 unit, Invitrogen), 1 × PCR buffer, MgCl2 (1.5 mM), dNTP (0.2 mM each), primers (0.2 μM each) and purified DNA from tissue (~100 ng). The primers 5′-GCTAAGAAGGCTGTTCCCTTCCAC-3′ and 5′-CTGGGTCATCTTTTCACGGTTGG-3′ amplify a 266 bp fragment from the β-actin gene using 35 cycles of 20 s at 94 °C, 20 s at 59 °C and 20 s at 72 °C. The primers 5′-GCAAATGGGCGGTAGGCGTGTA-3′and 5′-TCAGGGGGAGGTGTGGGAGGTT-3′ amplify a 966 bp fragment from pEGFP-N1 plasmid using 25 cycles of 20 s at 94 °C, 20 s at 62 °C and 60 s at 72 °C. The primers 5′-GCCTCATAGAACTGCCTGCGTGAGA-3′ and 5′-CCGCTTCCCCGACTTCCTTAGAGAG-3′ amplify a 351 bp fragment from pCMV-LUC plasmid using 25 cycles of 20 s at 94 °C, 20 s at 57 °C and 20 s at 72 °C. The PCR products were subjected to agarose gel electrophoresis in the presence of ethidium bromide and photographed using a GelDoc2000 (BIO-RAD).

2.2.7. Immunohistochemical analysis of tumor samples

Cross-sectioned tumors from euthanized mice were fixed overnight in freshly prepared pH-neutral formaldehyde (4%) followed by dehydration in 70% ethanol and embedded in paraffin. Four micrometer sections were prepared in a routine fashion on plus coated slides. The slides were deparaffinized, hydrated, and stained with H&E or using the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. Polyclonal rabbit anti-GFP (1:3500, Abcam) antibody was used to detect EGFP expression. Sections were counterstained with hematoxylin and mounted for microscope evaluations using an Olympus BX51 microscope (Olympus, Skovlunde, Denmark).

3. Results

3.1. SPLP preparation

Conventional lipids (cholesterol 55%, DSPC 20%, DDAB 15% and DSPE-PEG2000 10% and ^{3}H-CHE) were used for the preparation of SPLPs. The procedure was carried out at the 20 μmol total lipid scale using 200 μg reporter expression plasmid DNA. The plasmid DNA was prepared in-house using an endo-toxin-free GIGA plasmid kit. Almost complete DNA encapsulation was achieved in Tris-buffer at pH 7.0 by a combination of dropwise addition of ethanol to a final concentration of 40% and five cycles of freeze-thawing followed by extensive dialysis against HEPES buffer, pH 7.4 in order to remove the ethanol. At different stages in the procedure samples were isolated for agarose gel electrophoretic analysis (Fig. 1) [9] to estimate encapsulation. The sample in lane 2 constitutes 0.6% of a preparation after mixing of hydrated lipids and DNA and subsequent freeze-thawing. Some plasmid DNA is retained in extrusion filters, since the sample in lane 3 (0.6% of a preparation) isolated after extrusion through 100 nm filters has a lower staining intensity [19]. This could be due to DNA aggregation or semi-precipitation since several bands of DNA migrating higher in the gel than 5 kb are observed in this lane. After dialysis into HEPES buffer and removal of the ethanol only a very little amount of externally bound plasmid is seen migrating as a band of approximately 5 kb (lane 4). The remaining DNA staining is seen near the loading well indicative of liposome encapsulated plasmid. In lane 5 the sample has been digested with a mixture of DNase I and Exonuclease III [20], hence the free DNA, migrating as a 5 kb-band seen in lane 4 is lost, and only DNA protected in the liposome is seen near the loading well of the gel. Using standards of purified plasmid the PicoGreen assay was used to determine the DNA concentration in lane 4 and 5 and was estimated to be 18 μg DNA per 200 μl SPLP preparation used for one tailvein injection, an overall yield of 45% plasmid encapsulation. A tritium-labeled tracer lipid was added in

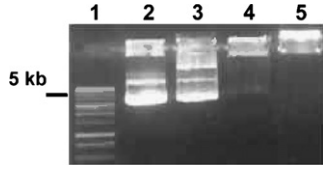

Fig. 1. Agarose gel electrophoresis/ethidium bromide staining of SPLP samples (0.6% of a preparation) in different stages of the preparation. Lane 1: DNA size marker, lane 2: SPLP before extrusion, ethanol added. Lane 3: SPLP extruded through 100 nm pore filters, lane 4: SPLP dialyzed into HEPES buffer, lane 5: SPLP dialyzed and digested with deoxynucleases to degrade externally bound plasmid DNA. Free plasmid DNA (migrating as ca. 5 kilobase fragment) and encapsulated DNA that is retained in the loading well.

the formulation and by scintillation counting we found that the recovery of lipids in the preparation was almost complete yielding 4 μmol total lipid in 200 μl buffer.

We decided to exploit the use of the SPLPs seen in lane 4 as the amount of plasmid DNA bound externally and released upon electrophoresis was very low. The SPLPs used for *in vivo* studies were characterized regarding their size and charge using dynamic light scattering. SPLP sample measurements yielded a narrow size distribution (1–3 nm) with an average of 144 nm ± 13 nm (standard error of mean, $n=3$), a low polydispersity index (0.1 ± 0.01) and a slightly negative zeta potential (−6.2 ± 1.3). These properties are favorable for long-term circulation in that the size should be low and not positive as this causes retardation in first-pass organs, whereas the neutral or slightly negative charge allows for long circulation time in the system [4].

3.2. Gene expression analysis in vitro

The dual properties of nanoparticles to be stably, long-circulating and at the same time yield transfection in target tissue require exploitation of the transfection properties. Dialyzed SPLPs containing pCMV-LUC plasmid (~0.8 μg) were added in full growth medium to tissue culture cells either easy to transfect, adherent lung cancer cells H1299 or hard-to-transfect suspension small cell lung cancer cells NCI-H69 and the luciferase reporter activity was analyzed two days later (Fig. 2). Compared to cationic lipoplex-mediated transfection [21] a moderate activity was measured in H1299 (14 ± 4 pg luc per mg protein) and a low activity was measured in NCI-H69 cells (0.7 ± 0.2 pg luc per mg protein). Since we recently showed that the pharmacokinetics of cationic lipoplexes were poor for systemic treatment of xenograft flank tumors [21], we decided to investigate the use of SPLPs in our xenograft tumor model. Furthermore, NCI-H69 tumor microenvironment may be very different from growth in suspension in culture media and this could potentially have a beneficial effect in relation to transfection from accumulating SPLPs.

3.3. Blood half life

The SPLPs with encapsulated plasmid DNA were tested *in vivo* using nude mice carrying xenograft flank tumors originating from human small cell lung cancer [13]. Animals were carrying 1–2 subcutaneous tumors on the flanks and used for experiments when tumors had reached 200–800 mm^3 in size. A typical preparation of SPLPs (1 ml) was used for 5 animals; each was injected with one dose of 200 μl intravenously (18 μg DNA, 4 μM lipids).

In Fig. 3 an experiment where blood was sampled by periobital puncture (eye vein blood) and analyzed for radioactivity by scintillation counting at different time points (0.25, 2, 5, 22 and 29 h) after injection. An exponential clearance from the blood was measured and a log–linear regression analysis yielded a half life estimate, $T(1/2)=10.8 \pm 0.1$ h (average ± standard error of the mean). The half life was determined from pooling three independent experiments, including a total of 13 mice and sampling up to 48 h after injection.

3.4. Luciferase reporter gene expression in vivo

After having received a single intravenous injection of SPLP, mice showed normal behavior and were euthanized after 24 h and organ samples from tumors, heart, lung, liver, kidney and spleen were collected. The luciferase activity in protein extracts was assayed, and expressed as pg luciferase per g total protein (Fig. 4). Two independent experiments were pooled combining data from a total of 12 mice carrying 21 tumors in total, since some mice had only one flank tumor. Assay background was established previously [21] from control animals and determined to be 10 pg luc per g protein (indicated with dotted line). Interestingly, luciferase activity was found almost exclusively in tumor tissue (52 ± 15 pg luc per g protein), only a small activity above background was found in the lung (19 ± 12 pg luc per g protein), although considerable variation between mice was observed.

3.5. Biodistribution of radioactive lipid and detection of plasmid DNA

Having measured the blood half life of tracer lipid we wanted to measure the biodistribution in various organs and the xenograft flank tumors. After either one or two days the mice were euthanized and tumors, heart, lung, liver, kidney and spleen tissues were sampled and homogenates were subjected to scintillation counting (Fig. 5). Data for each time point was combined from two independent SPLP experiments, where $n=4$–7 for each experiment and time point. The amount of radiolabel measured in samples was extrapolated to whole organ accumulations and expressed relative to the input dose given by intravenous

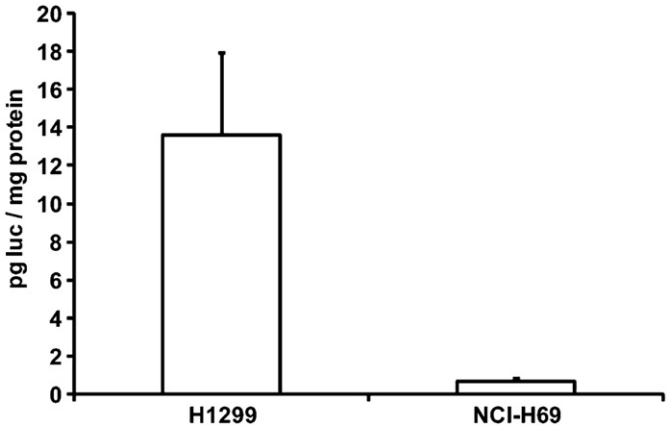

Fig. 2. Reporter gene expression by transfection of NCI-H69 (SCLC) and H1299 (NSCLC) cells *in vitro*. Luciferase activity in cell lysates was measured two days after transfection and expressed as picogram luciferase per milligram protein. Data from at least three independent experiments with different SPLP preparations were collected and the average and standard error of the mean are given.

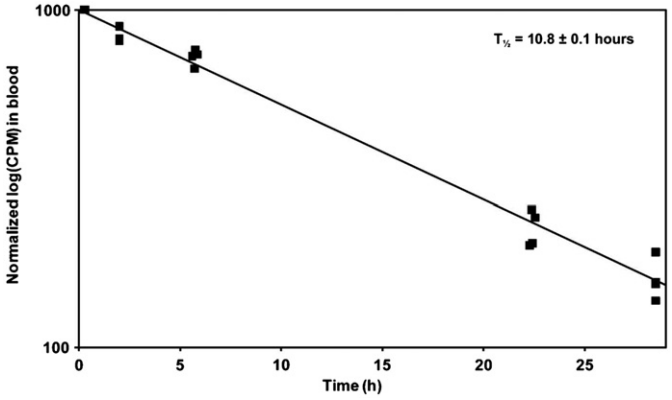

Fig. 3. Blood availability of tritium-labeled SPLP. Following tailvein injection of SPLP blood samples were drawn by periobital plexus puncture (eye vein blood) after 15 min, 2 h, 5 h and 22 and 29 h and the amount of radioactivity was quantified by scintillation counting. After log-transformation and normalization a linear model was applied to each mouse data set ($n=4$) estimating the halftime that correspond to a simple exponential clearance. The half life was determined from combining three independent experiments ($n=4$–5) and the average and standard error of the mean are given.

injection. Furthermore counts per minute (CPM) were normalized to the amount of tissue analyzed and the biodistribution was expressed as CPM per gram tissue sample. Interestingly, already after one day we found $10.0 \pm 1.8\%$ of the injected dose (average $\pm$ SEM) in the tumor tissue, whereas only smaller amounts $1.8 \pm 0.3\%$, $2.4 \pm 0.4\%$ and $3.7 \pm 0.5\%$ were found in heart, lung and kidney tissue, respectively. Entrapment by the reticuloendothelial system was evident, since almost half ($41.9 \pm 9.4\%$) of the tracer lipid resided in the spleen, presumably due to uptake by monocytes and macrophages [4,22] and $16.8 \pm 1.5\%$ was found in liver, presumably due to uptake by Kuppfer cells. After two days, although the measured radioactivity was lower, a high relative accumulation in tumor tissue ($4.2 \pm 0.9\%$) persisted and a shift from spleen (11.0 ± 0.8) towards liver (12.3 ± 3.3) and kidney (3.4 ± 1.0) was observed, while the amount in heart and lung remained much lower. When considering the distribution of radioactivity in the measured samples only, around 20% of the radiolabel found in the samples was present in the tumor tissue (Fig. 5, right part).

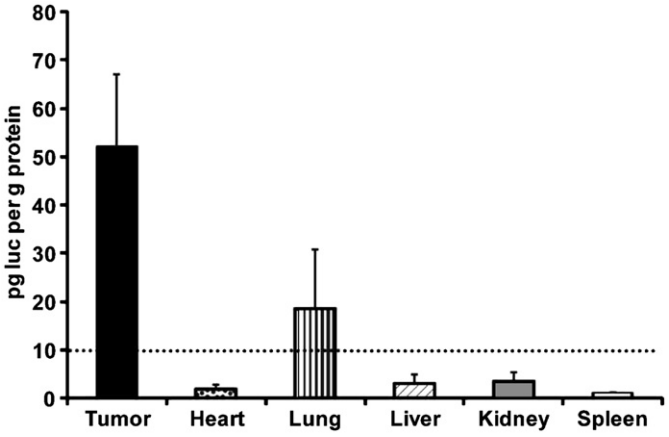

Fig. 4. Luciferase reporter gene expression *in vivo*. 24 h after receiving a single intravenous injection with 200 μl SPLP, mice were euthanized and organ samples collected and the luciferase activity in protein extracts was assayed, here expressed as pg luciferase per g total protein. Two independent experiments were pooled combining data from a total of 12 mice carrying 21 tumors in total, since some mice had only one flank tumor. Assay background was at 10 pg luc/g protein (indicated with dotted line). Only tumor and lung tissues showed mean activities above background, however, with high variation. Error bars indicated the standard error of mean.

In order to confirm that plasmid DNA was distributed similarly, DNA was extracted from tissue samples and subjected to semi-quantitative PCR analysis. Fig. 6 shows the result from analyzing two representative mice injected with SPLPs containing either pEGFP-N1 (A) or pCMV-LUC (B) plasmid. Upper panels show that plasmid is detectable in all tissue samples and that the amount of PCR product is in good alignment with the distribution of radioactive lipid in the different organs as shown in Fig. 5. Lower panels show control amplification of a chromosomal DNA fragment from beta-Actin.

3.6. Immunohistochemical staining against EGFP reporter in tumor tissue

We examined the reporter gene expression in xenograft tumors of mice by immunohistochemical staining. One day after intravenous injection of SPLPs containing pEGFP-N1, mice were euthanized and tumor tissue analyzed. Fig. 7 shows tumor sections stained with an antibody recognizing EGFP, hence no signal is observed in the left panel (A), where the mouse did not receive a liposome dose. A strong signal in discrete cells is observed in the right panel (C), where the mouse received an intratumoral injection of recombinant adenovirus expressing EGFP [13]. However as shown in the middle panel (B) very low to undetectable levels of EGFP were observed in tumors from mice injected with SPLPs containing the pEGFP-N1 plasmid.

3.7. Tumor growth in response to treatment with suicide gene therapy

We found that the blood circulation time and biodistribution was independent of the cargo plasmid used and hence we performed an

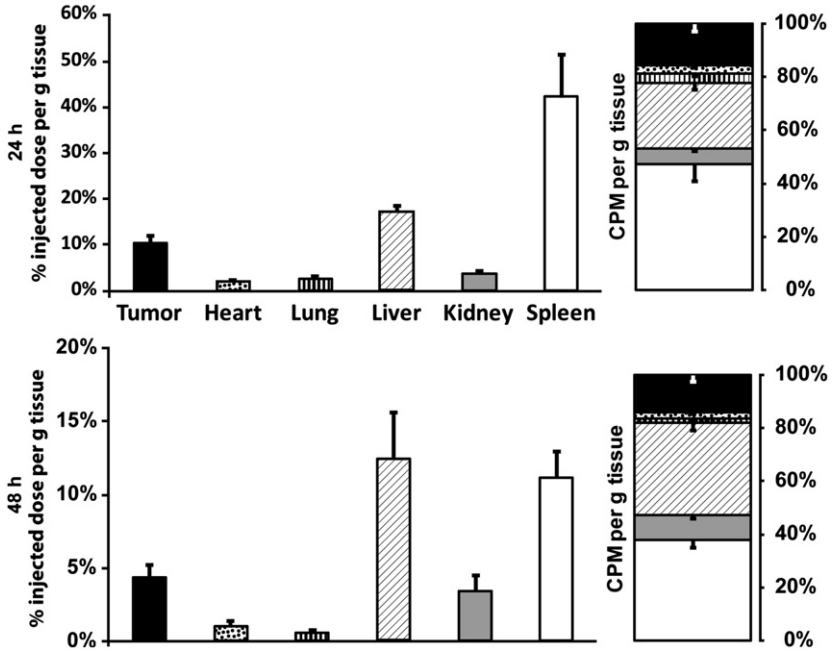

Fig. 5. Biodistribution of radioactive tracer lipid. The distribution in tumor, heart, lung, liver, kidney and spleen total organs was calculated relative to the injected dose after 24 h (top panel) and 48 h (lower panel). The relative distribution of tritium counts per minutes per gram tissue sample is shown to the right. For each time point two independent experiments (n=4–7) were pooled and averaged and error bars indicate standard error of the mean.

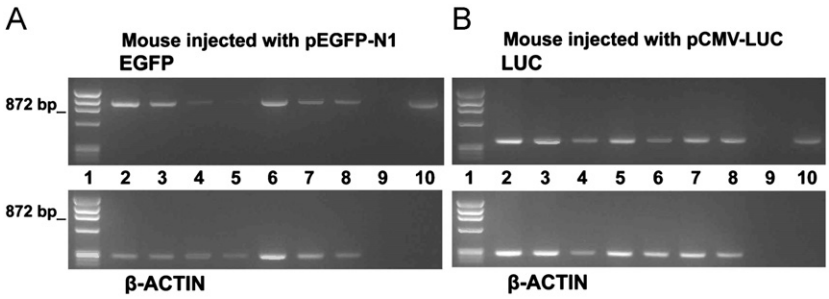

Fig. 6. Detection of plasmid DNA in tissue samples. Tumor and mouse organ homogenates originating from 5 to 10 mg tissue were subjected to DNA purification and the resulting mix of chromosomal and plasmid DNA was subjected to semi-quantitative PCR analysis. A primer pair amplifying the β-actin gene was used as control (lower panels). Representable samples from a mouse injected with (A) pEGFP-N1 encapsulated liposome was analyzed with EGFP specific primers and (B) pCMV-LUC encapsulated liposome was analyzed with LUC specific primers (top panels). Agarose gel lanes were loaded with 1: φX174 *Hae*III-digested DNA, 2–8: PCR template was DNA from left-flank tumor, right-flank tumor, heart, lung, liver, kidney and spleen, respectively, 9: blank control without DNA, 10: purified plasmid.

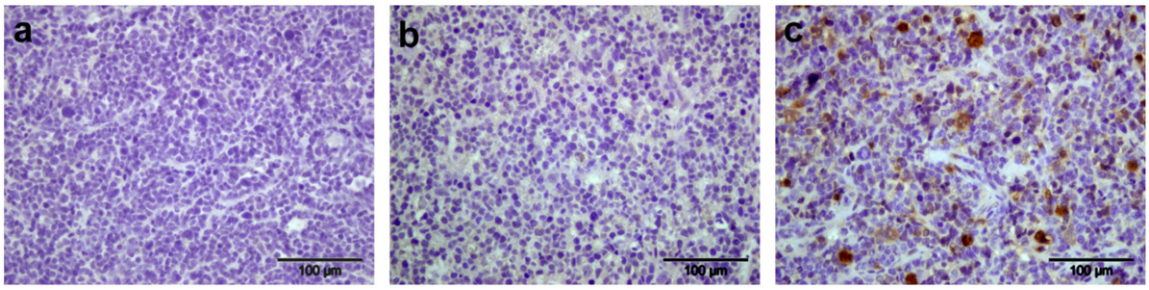

Fig. 7. Immunohistochemical analysis of EGFP expression in xenograft tumors treated with SPLP encapsulated with pEGFP-N1 plasmid, two days after intravenous injection. Tumor tissue sections were stained with anti-EGFP antibody (brown color) and counterstained with hematoxylin and eosin, Left panel (a) is from an untreated tumor, middle panel (b) is from a tumor where the mouse received intravenous SPLP injection and in the right panel (c) the tumor was directly injected with a recombinant virus expressing EGFP. The scale bar indicates 100 μm.

initial study of intravenously delivered suicide gene therapy as we recently reported to be useful for SCLC [13]. The SPLPs containing pINSM1-SCD-FLAG, a plasmid expressing a FLAG-tagged variant of "super cytosine deaminase" from a SCLC-specific INSM1 promoter, was intravenously injected and followed by an intraperitoneal injection of the non-toxic prodrug 5-fluoro-cytosine. Only in cells expressing the delivered plasmid this compound is converted to the toxic 5-fluoro-uracil. Using caliper-measurements, we monitored the tumor growth in mice that received the prodrug and compared to mice that did not (Fig. 8); however, we did not observe a difference in growth between the two groups after two days. Using immunohistochemical staining we analyzed tumor tissue sections from injected animals for FLAG-tag positive cells [13] indicating expression of suicide gene and applied a TUNEL assay to visualize cell death. We were unable to detect the expression of suicide gene product presumably due to limited transgene expression in accordance with luciferase experiments. No significant increase in the fairly high apoptotic index in the tumor tissue could be observed as a result of 5-fluoro-cytosine treatment (data not shown). Hence we noted that the effect of transgene expression was diminishable, although the accumulation of SPLPs in tumor tissue remained high.

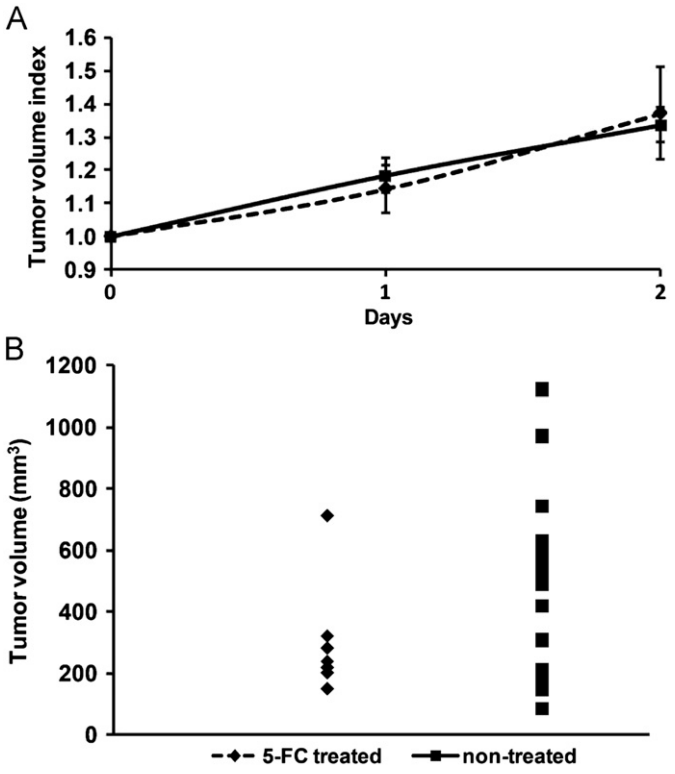

Fig. 8. Tumor growth after intravenous administration of SPLPs containing SCLC-target suicide gene therapy. Top panel (A) shows indexed tumor growth with and without 5-FC administration and lower panel (B) shows initial tumor volume. Diamonds represent 5-FC-treated tumors ($n=7$, 4 animals) and squares represent non-5-FC-treated tumors ($n=15$, 9 animals).

4. Discussion

4.1. Stabilized plasmid lipo-particles

The importance of nanoparticle properties that on one hand ensure systemic stability by having a PEG layer on the surface and on the other hand are able to transfect cells with great efficiency once the nanoparticle has arrived at its target site has been discussed in many papers (e.g. Hatakeyama et al. [23]. One of the

most promising strategies involve stabilized plasmid lipo-particles [10] using custom-designed lipid components including PEGylated [24] and cationic lipids [25]. Here we tested a formulation of similar properties from commercially available lipids to assess nanoparticle properties, systemic stability, transfection efficiency and usefulness in a suicide gene therapy application.

We have previously described the efficient encapsulation of plasmid DNA into PEGylated immuno-liposomes with 70% of the plasmid being encapsulated in the interior [9] and while using similar procedures we obtained SPLPs in approximately 150 nm in size.

In addition to PicoGreen assay, agarose gel electrophoresis provide a method for evaluating the encapsulation and externally bound plasmid DNA [9,15,16], and hence we found that plasmid DNA was effectively encapsulated and protected from nucleases (Fig. 1) and could be applied to cells and animals without further purification.

A nuclease digestion and subsequent purification by size-exclusion chromatography did not change the size of the particles, but merely caused an unfavorable dilution of the SPLPs. Furthermore, we measured unchanged luciferase activity *in vitro* when analyzing nuclease-treated SPLPs; however, concerns of systemic immune responses to nucleic acids [26,27] or C-p-G [28] *in vivo* persist and could favor additional steps of SPLP purification [9,20].

4.2. Transgene expression

When we analyzed the luciferase activities of SPLPs with encapsulated luciferase reporter plasmid we found considerable activities in human non-small cell lung cancer H1299 cells. In small cell lung cancer NCI-H69 cells the luciferase activity was much lower (Fig. 2). Presumably, this difference reflects growth properties and internalization capabilities of the two cell lines and has been found with a number of different lipid-based transfection reagents [13,21] (unpublished data). Nevertheless xenograft tumors derived from NCI-H69 cells growing on the flank of nude mice could be transfected with our SPLPs by intravenous delivery, as we measured a moderate reporter activity (Fig. 4) comparable to the results of others [10]. This finding could relate to the fact that tumor cells that are actively dividing have fewer intracellular barriers to successful SPLP-mediated transfection than other diffentiated tissues analyzed.

Furthermore, in lung tissue we measured luciferase activity above background level, although only a low amount of SPLP resided in the lung (2–3%, Fig. 5). It could be speculated that a population of cells in lung capillaries is relatively easy transfected by lipo-particles and theoretically examination by *in situ* immunohistochemical methods would elucidate this phenomenon. Additionally, a careful *in situ* analysis of transgene expression may shed light on the location of cells within the tumor that are successfully transfected by this route of delivery [4] and degree of extravasation from tumor vasculature as it has been reported in case of DOXIL® [29,30].

However, when attempting to detect EGFP by immunohistochemical staining after intravenous injection of SPLPs with encapsulated pEGFP-N1 plasmid, we only found very weak or non-detectable expression in tumor (Fig. 7) and lung tissue sections (data not shown), hence it is suggested that protein levels are too low for positive immuno-detection.

4.3. Systemic stability and biodistribution

It has been established that inclusion of a PEG-modified lipid in the formulation facilitates long systemic circulation time and may circumvent immunostimulation and rapid clearing from the system [31], although recently concerns have been raised regarding immune responses [26]. Prolonged circulation time of liposomes leads to accumulation at the site of disease, the so-called enhanced permeability and retention (EPR) effect, presumably due to leaky endothelial lining in the blood vessels and impaired lymphatic drainage [6]. For a strategy involving gene therapy against a disseminated cancer EPR ameliorates the transfection perspective profoundly; not all cells in the body need to be transfected, hence a targeted gene medicine can be greatly assisted by ensuring that the circulation time is long enough for the accumulation in cancer tissue to occur. In the SPLP formulation we included 10% DSPE-mPEG2000, which is the PEG–lipid in the DOXIL® formulation that ensures a very long circulation half life of 16–30 h in mice [4,32,33].

The SPLPs were prepared with a non-degradable, non-metabolizable radioactive lipid label in the formulation enabling the easy evaluation of biodistribution by scintillation counting of samples upon injection of the SPLPs into mice [18]. Hence by blood sampling in the time after SPLP injection we measured a blood half life of more than 10 h allowing sufficient time for the EPR effect to work [34]. Previous work has shown that blood plasma half life of 6–7 h is sufficient for tumor accumulation of the particles [10,11]. Biodistribution of radioactively labeled SPLPs was calculated in two different ways. Firstly, the distribution was calculated considering the total weights of the analyzed organs relative to the injected dose, and a clearing from the system was found over two days with increasing accumulation in liver and kidney, while a relatively large dose was retained in tumor. Secondly, the radiolabel distribution in the isolated tissue samples was calculated, and here around 20% of the radioactive lipid resided in tumor tissue one and two days after intravenous administration. Predominantly the remaining radiolabel appeared to have been taken up by the reticuloendothelial system, macrophages and monocytes in the spleen and Kuppfer cells in the liver [22] and importantly no accumulation in first-pass organs was observed [21].

In addition to tracking the radioactive lipid label we also performed a PCR analysis of tissue samples in order to verify that the gene cargo of liposomes was present in the different organs. Indeed we found that both luciferase and EGFP expression plasmids were detected in a semi-quantitative assay in good alignment with the distribution of the radioactive lipid label (Fig. 6).

4.4. Suicide gene therapy

In our strategy to develop a suicide gene therapy for small cell lung cancer the most promising system to date is the yeast cytosine deaminase (YCD) gene fused with the uracil phosphoribosyl transferase (YUPRT) gene driven from the human INSM1 promoter in combination with administration of 5-fluoro-cytosine (5-FC) prodrug [13]. When we tailvein-injected tumor-bearing nude mice with suicide gene encapsulating SPLPs and administered prodrug intraperitoneally in two preliminary experiments, we could neither observe a significant reduction in tumor size by caliper measurements (Fig. 8) nor an increase in dead tumor cells measured by TUNEL-positive cells in fixed tissue sections. A high apoptotic index of the cancer cells within the tumor could be masking a subtle effect of the suicide gene therapy treatment ([13], data not shown). Previous trials with this suicide gene system utilizing intratumoral delivery showed a prompt response in tumor growth already after one or two days [13], hence these results are in alignment with the low efficiency of transgene expression as described above using luciferase and EGFP reporters.

Even so, the system constitutes an attractive delivery vehicle that enables tumor targeting after systemic administration without causing adverse retention in non-target organs allowing evaluation of cancer gene therapy strategies within the appropriate tissue of a xenograft tumor model. Obviously the transfection efficiency in target tissue requires augmentation of the present results with

commercially available lipids and optimization of lipids formulated into SPLPs is ongoing [25,35]. Furthermore we are aiming to incorporate lipids responsive to local tumor environment, e.g. using pH-sensitive, detachable PEG-moieties [36] or other enzyme activities found in tumor environment [37] and hereby arriving at a transfection efficiency that is useful in a therapeutic setting.

5. Conclusion

A protocol is described for the preparation of SPLPs with encapsulated plasmid DNA for treatment of SCLC using a transcriptionally targeted suicide gene therapy approach. The system was tested for systemic delivery to xenograft tumors in nude mice and showed attractive properties of circulation and tumor accumulation, however without causing effective transfection. The methods will be useful for investigators, who wish to extend *in vitro* studies of controlled release of nucleic acid cargo from SPLPs into cancer cells to xenograft model tumors *in vivo* for example by incorporating bioresponsive components that change the properties of SPLPs due to local enzymatic activities or pH.

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